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Hyaluronic acid with or without bone marrow derived-mesenchymal stem cells improves osteoarthritic knee changes in rat model: A preliminary report

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Despite being a complex degenerative joint disease, studies on osteoarthritis (OA) suggest that its progression can be reduced by the use of hyaluronic acid (HA) or mesenchymal stem cells (MSC). The present study thus aims to examine the effects of MSC, HA and the combination of HA-MSC in treating OA in rat model. The histological observations using O'Driscoll score indicate that it is the use of HA and MSC independently and not their combination that delays the progression of OA. In conclusion, the preliminary study suggest that the use of either HA or MSCs effectively reduces OA progression better than their combined use.

Keywords: Hyaluronic acid, Mesenchymal stem cells, Monosodium iodoacetate, Osteoarthritis rat model

Osteoarthritis (OA) is a degenerative joint disease characterized by the progressive reduction of extracellular matrices (ECMs) in joint cartilage and bones. This process ultimately leads to joint destruction[1]. The prevalence of OA increases with age, affecting a large proportion of all people above the age of 65 years[2-4]. Established treatments for OA other than total knee replacements are mainly preventive which include weight control, exercise or treatment of underlying metabolic diseases. Total knee replacement also has its limitations including, increase in wear rates and inability for full knee function (for eg. squatting and kneeling). More recently, neutraceuticals which contain polyphenolics, have also been used for treating OA[5-8]. Although these treatments for OA have been well described in many literatures[9], these therapies have failed to halt the progression or reverse the damage caused by OA[10, 11].

Due to the increasing incidence of OA coupled with inefficient therapeutic choices, novel cartilage repair strategies are in need. Owing to its self-renewal capacity and their potential for chondrogenic differentiation, Mesenchymal stem cells (MSCs) have been considered a potential cell source for cartilage tissue engineering. MSCs also possess potent immunosuppressive and anti-inflammatory effects[12]. In addition, MSCs produce bioactive factors that initiate endogenous regenerative activities in OA joints. Murphy *et al.*[13] have reported that the use of MSCs in goat OA knees regenerate damaged cartilage. From this study, it appears that the injected MSCs increase the metabolic activities of endogenous progenitor cells through various direct or indirect interactions which also regenerate meniscus. This retards the progress of cartilage degeneration thereby relieving the symptoms caused by OA[13].

Intra-articular (IA) hyaluronan (HA) is widely prescribed by medical practitioners as a symptom-modifying treatment to improve pain associated with OA of the knee. However, there is substantial evidence suggesting that HA in certain patient populations may provided disease-modifying activity[14]. Exogenous HA induces endogenous HA synthesis, possibly by stimulating the regenerative process within the joint[15]. It is also been suggested that HA has structure-modifying actions and may therefore provide benefits in the physical repair of damaged cartilage. However, this effect is still a matter of debate as there is minimal evidence to support the role of HA in modifying OA[16].

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Direct intra-articular injection of autologous MSCs in a dilute solution of HA injected into the knee joints of OA in goats have demonstrated marked regeneration of the medial meniscus. Whether the changes observed in these MSC-treated joints are the result of direct tissue repair by the transplanted cells or from their interaction with host synovial fibroblasts at the site of injury remains unclear[13]. Lee *et al.*[17] postulated that hyaluronic acid might facilitate the migration and adherence of MSCs or MSC-like cells, most likely present in the synovium, to the defective sites. In their study, it has been demonstrated that the use of HA with MSC has produced positive outcomes; however Lee *et al*. did not compare the effects of MSC without the presence of HA. Interestingly, extensive literature search has also not demonstrated any studies which determines the effect of MSC or HA on OA in a side-to-side comparison.

Considering that both MSC and HA have shown to improve OA, it is assumed that, the combination of these two would result in even superior cartilage healing. A study is therefore conducted with the aim to investigate whether the combination of MSCs and hyaluronic acid may provide an additive or enhanced effect as compared to their individual potential components in treating OA.

Materials and Methods

Animals—Eight weeks old matured male Sprague-Dawley rats (36) weighing between 100-150 g were obtained from the Experimental Animal Centre of University Putra Malaysia. All animal experiments have been conducted according to guidelines for animal handling and welfare in University of Malaya. The present study has been approved by University Malaya ethics review committee for animal research (OS/05/08/2009/WJH(R)).

OA induction—MIA injection (Crystal Powder M=185.96 g/mol, Germany, Sigma; 2 mg) was prepared under sterile condition. Rats were anesthetized using ketamine hydrochloride (50 mg/mL for injection USP, Rotexmedica, Germany) and xylazine hydrochloride (Ilium Xylazil-20 20 mg/mL for injection, Troy laboratories PTY limited). The dose was measured and delivered according to the rat(s) body weight {30X rat weight/100 and 3X rat weight/20}. After 15 min rat(s) were placed in supine position. Intra-patellar tendon was identified and a single intra-articular injection of 2 mg of monosodium iodoacetate (MIA) in a total volume of 25 µL was injected into the right knee. The dose of MIA has been chosen based on previous literature[18,19]. Right knees of the rats were given a single intra-articular injection of 25 µL 0.9% normal saline.

Bone marrow-mesenchymal stem cells (BM-MSCs) preparation—MSCs isolation was carried out according to the methods previously described by Gnecchi and Melo[20]. Mesenchymal stem cells were isolated from the femur of 4 rats which were euthanized. The adherent cells at passage 2 were detached by incubating with 0.25% trypsin–EDTA solution (Invitrogen/GIBCO) for 5–10 min at 37 ºC. Low glucose DMEM medium was added to inactivate the trypsin. The cells were centrifuged at 1900 rpm for 10 min and the pellet was re-suspended in 1 mL complete medium for use in the treatment groups.

Administration of BM-MSCs and HA—At day 18 following the induction of OA, the rats were treated with HA and BM-MSCs. In group I (n=7) no treatment was administered for the right knees (control group). In the remaining three groups, two successive intra-articular injections were administered a week apart. In Group II, intra-articular injection of 25 µL of HA (Hyalgan® sodium hyaluronate; Fidia Farmaceutici S.p.A., AbanoTerme, Italy) was administered using a 27 gauge, 0.5 inch needle. For Group III and Group IV, BM-MSCs ($3\text{-}5x10^6$ cells) and a combination of BM-MSCs and HA were injected respectively.

Animal sacrifice—At day 18, one rat from each group was sacrificed to determine the degree of OA (Grade O–IV) created following MIA, while the remaining rats were continued for the experiment. Six weeks following the administration of the first intra-articular injection, all remaining rats were sedated, euthanized and sacrificed. Both knees were dissected and the joints exposed. Distal femur was dissected at the supracondylar level while tibia was dissected below the level of tibila tuberosity. Exposed joint surfaces were visually examined following which the tissue was sent for histological analyses.

Macroscopic Evaluation—Two independent examiners who were blinded to the study groups were recruited to examine the knee joints of rat sacrificed at 18 and 60 days of the study. The joints were photographed, recorded and assessed according to Collins and McElligott[21] grading system. Values used for the analysis is the average obtained, or when not possible, the worse of the two. The grading system is solely descriptive or at best semi-quantitative as it embodies the use of categorical (ordinal) data.

Histological evaluation—The samples were decalcified in 5% formic acid. The formic acid was changed every 2-3 days for a total period of two weeks. The resulting histological sections included femoral condyles, tibial plateaus and menisci. For the normal contralateral knee, a single section was prepared. Prior to staining, the paraffin was removed by immersing the slides for two minutes in two xylene and two ethanol jars. Serial sagittal sections (6 µm thick) were prepared and stained with haematoxylin and eosin (cellular architecture), toluidine blue and Safranin-O (proteoglycan contents of matrix). The severity of articular cartilage lesions was graded, using the histological grading method based on the modified O' Driscoll score. This score assesses structural integrity (0–2 points), surface regularity (0-2 points), cellularity (0–3 points), matrix staining (0–3 points), nature of predominant tissue (0–3 points), and chondrocyte clustering (0-2 points) in total and, has a maximum score of 15 points. A low score indicates severe damage.

Statistical analysis—One-way analysis of variance (ANOVA) was employed. Post hoc analyses using T-Test with Bonferroni correction were performed if significant values were obtained. Pearson Chi-square test was used to analyze the categorical data. Significance was set with P values < 0.05. All analyses were conducted using the statistical software SPSS version 14.0.

Results

Macroscopic observation—At 60 days post-OA induction, the cartilage on the left femoral condyles (normal knee) was in pristine condition. Surface cartilage was glistening white and smooth, with no defects or osteophytes observed (Fig. 1A). In the right knees of all the rats, characteristics of OA including fibrillation, erosion, and osteophyte formation especially in the medial femoral condyles were present. OA changes ranged from mild to severe (Fig. 1). Gross morphological assessment performed using Collins descriptive grading system revealed distinct variations between the groups. However, these values were not significantly different (Chi-Square test: P=0.853). The mean distribution of the grades according to the different treatments is presented in Fig. 2. It is worthy to note that in using the Collins score, the lower the value the better the tissue quality.

Histological grading and analysis—The specimens were stained and scored based on the O'Driscoll scoring system. The score ranged from 0-15, with the lower score denoting a poorer outcome. Cartilage tissues from the left knee (normal knee) showed normal histological appearance and proteoglycan content at the end of 60 days (Fig. 3A).

Figure 4 shows the distribution of O'Driscoll scoring by different treatment groups at 6 weeks post treatment. The untreated group (Fig. 3B) had the lowest score (1.9 ± 1.9), reflecting the poor cartilage tissue quality observed in severe OA conditions. There were full thickness loss of articular cartilage, loss of tissue integrity and, severe hypo-cellularity

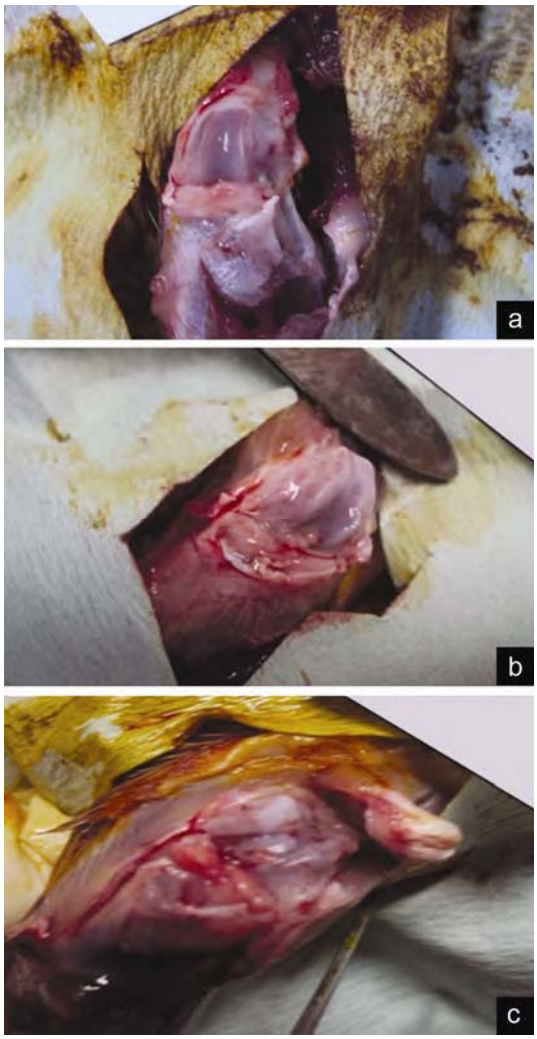

Fig. 1—Different grades of OA are compared. Each of the treated group had normal knee joint morphology. This was further confirmed using histological analysis (A). Macroscopically, in Grade II OA knee, surface irregularity and deep fibrillation with loss of glistering appearance is observed. However, no osteophytes were present (B). Severely damaged left knee joint: loss of articular cartilage (denuded bone) and marked osteophytes are present (C).

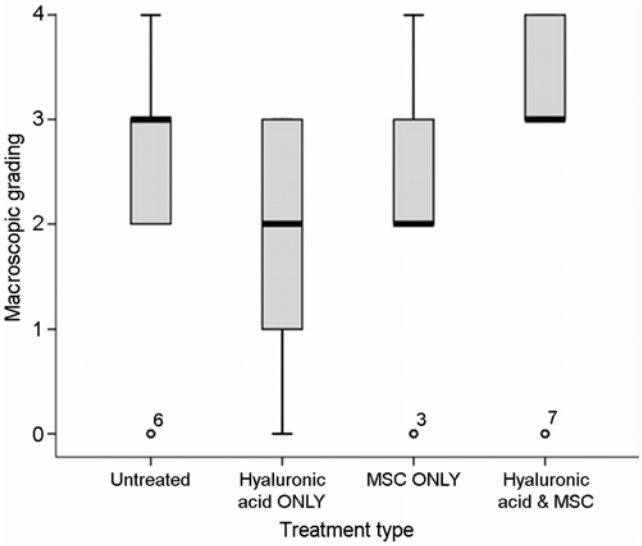

Fig. 2—Box plot of the distribution of the results obtained using Collins and McElligott's score by treatment groups. It is observed that the untreated and M&H groups had the highest median score which reflect poor results, while the HA and the MSC intervention group showed lower median score indicating that the cartilage tissue was better. Note: the higher the median score, the poorer the results.

with markedly reduced staining uptake. In contrast, the HA group had the highest mean score (8.3±1.9) as demonstrated in Fig. 3C. The preserved structural integrity of the continuous surface, intact tidemark, minimal hypo-cellularity and a minimal reduction of Safranin-O (Saf-O) were all observed in this group. The scores of the MSC treated group Fig. 3D was marginally lower to the scores in the HA group (7.3±2.0). The histology results for this group showed preserved structural integrity with no fissuring and reduced staining was evident. Cloning of cells and loss of cellular arrangement were also observed. The combination of MSCs and HA (Fig. 3E) which was thought to provide superior outcome, did not produce the higher scores that was expected (5.6±2.1). The statistical analyses performed (ANOVA) demonstrated that at day 60, there were significant differences between these groups (P=0.01). Using the post-hoc test with Bonferroni correction, significance was only observed between the untreated and treated groups.

To determine the effects of treatment on the progression of OA, the scores at different time points were measured. By comparing the scores at day 0, 18 and 60, it was observed that there is a steady decline in the O'Driscoll scores in the untreated group. With treatment at day 18, the rate of decline in the O'Driscoll scores in the HA, MSC or HA+MSC was lower indicating that the intra-articular injections had slowed down the rate of cartilage degeneration in rats (Fig. 4). Statistical analyses (One-way ANOVA) were done to compare the results between day 0, 18 and 60. Significant differences between the different time points were observed in all the groups. Comparisons between days 18 to 60 were of interest as this demonstrates the significant effect of intervention. Post-hoc test with Bonferroni correction was performed for each of the group. There was no significant difference in the HA treated group (P=1) and the MSC treated group (P=0.18). However, there was significant difference in the control group (P<0.001) and, HA-MSC treated group (P=0.03), which indicates that the combined effect of HA and MSC failed to slow down the progression of OA.

Discussion

This present study was performed to investigate the efficacy of MSC, HA and the combination of HA-MSC in treating OA using rat model. Administration of two courses of HA and MSC resulted in significant reduction of OA progression at 6 weeks after MIA induction. It was also demonstrated that HA or MSC produced improved healing when compared with the control group. This beneficial effect was especially apparent with regards to preventing the degradation of cartilage at the tibial plateau. Unexpectedly, the HA-MSC-treated group only showed partial healing of cartilage both histologically and morphologically. This result was further supported by the Posthoc T-tests when the progression of OA was compared between day 18 and day 60. The morphological (Collins) results in this study did not demonstrate statistically significant changes as those observed in the histological (O'Driscoll) scores. This may have been due to the poor sensitivity in the technique employed using this method of grading. Especially when considering that the rat joint may be too small for an effective assessment to be performed. This is reflective from the large variation seen between the observers' scores when using the Collins scoring system.

Although the mechanism facilitating the improvement of OA following the intra-articular injection of HA is still unknown, it has been postulated that the restoration of the elastoviscous properties of synovial fluid may be the most likely explanation[15]. It is also postulated that intra-articular injection of HA may induce endogenous HA secretion by the synovial cells, a process called

visco-conductive effect, thereby increasing the viscocity of the joint fluid[15]. Other possible mechanism could include the chondro-protective effect which is created when HA is combined to aggrecan monomers. This results in the formation of highly negatively-charged aggregates which absorbs water and thus increasing the resiliencey of cartilage. The biochemical activities of hyaluronic acid may serve as an inhibitor to leukocyte activities and as the result prevents the cumulative effect of ongoing inflammatory process that would otherwise cause joint destruction[22,23].

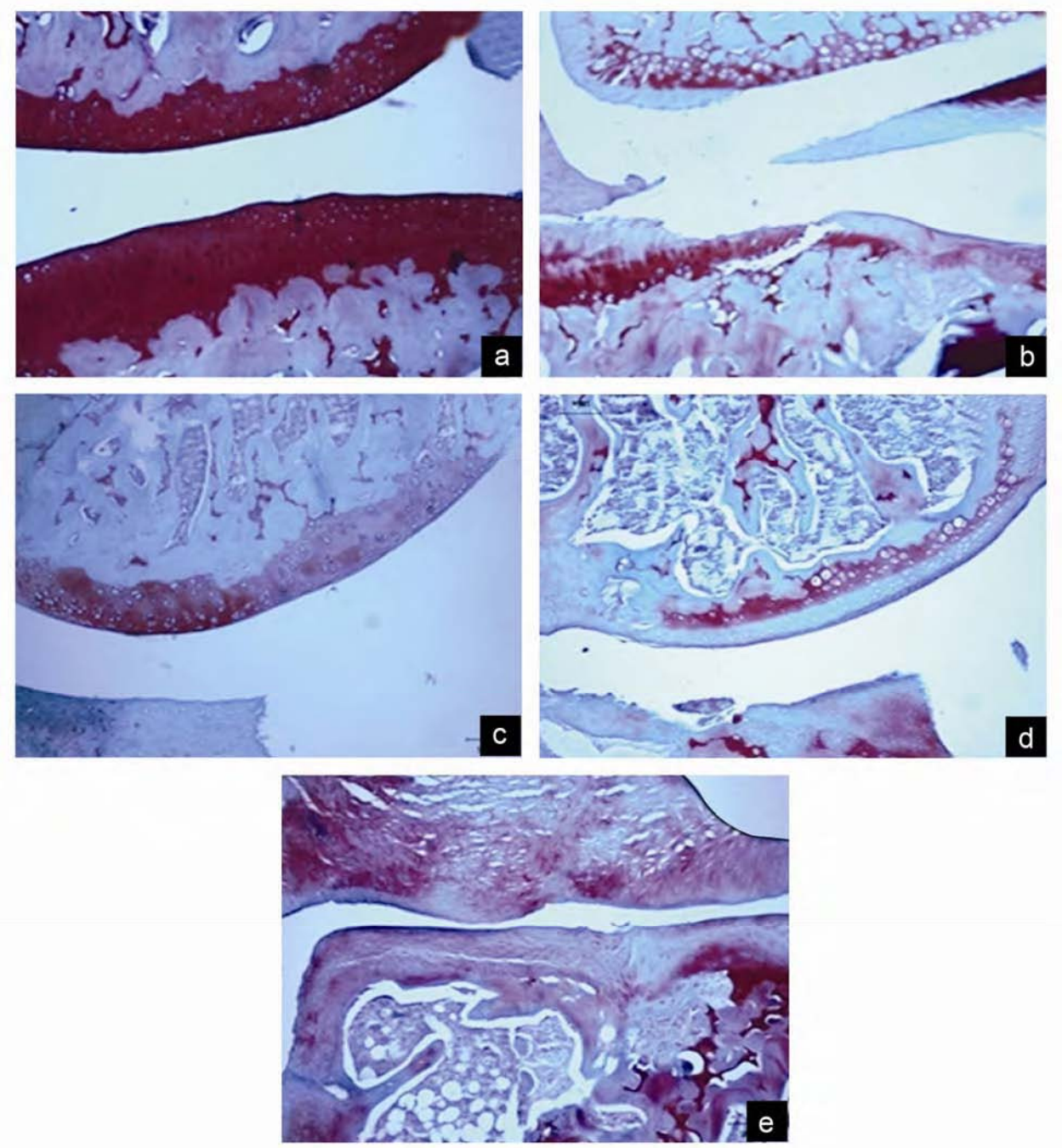

Fig. 3—O'Driscoll scoring system with varying degree of OA. The slide (10X) elaborates the articular cartilage of the (A) normal rat: structural integrity, normal cellular distribution and orientation, normal staining with Saf-O and intact tidemark, (B) Untreated: full thickness loss of articular cartilage, loss of integrity, severe hypocellularity and markedly reduced staining is seen. (C) HA treated, (D) MSC treated and, (E) HA-MSCs treated.

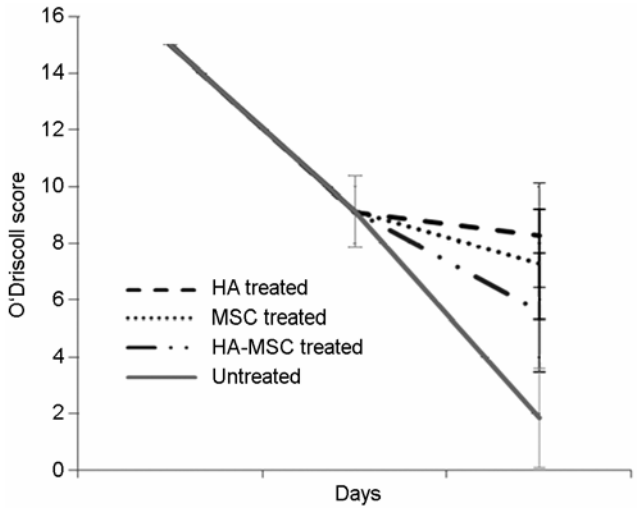

Fig. 4—Line plot for O'Driscoll score in different treatment groups at different time points of the study. The use of either HA, MSC or HA+MSC appears to reduce the rate of cartilage degradation (untreated). Error bar=±SD.

Goldberg and Buckwalter's[14] in their meta-analysis concluded that there is clinical evidence to support that in addition to relieving the symptoms of OA, hyaluronic acid also modifies the diseased cartilage and the rate of OA disease progression. Jubb *et al.*[23] demonstrated that three courses of three weekly injections of Hyalgan® (a high molecular weight synthestic hyaluronic acid) significantly reduced the progression of joint space-narrowing in the subset of patients presenting with the most severe signs at the start of the study. Another open-label and steroid-controlled study conducted by Frizziero *et al.*[24] demonstrates that one course of five weekly Hyalgan® injections produced significant reconstitution of the cartilage, improved the chondrocyte metabolism, and reduced the inflammation process within the synovial membrane. Although these short-term clinical benefits seen with Hyalgan treatment are encouraging, they require confirmation in well-controlled long-term studies. Nevertheless, in the management of a chronic and progressive disease such as OA, long-term pain relief to the extent reported here with Hyalgan, coupled with a good tolerability and safety profile, represent important attributes for pharmacologic intervention[25,26].

The use of MSC injection into joints to treat OA has been previously described. According to Caplan[27], MSCs could be used as trophic producers of bioactive factors to initiate endogenous regenerative activities in the OA joint. In the present study, after 6 weeks from the introduction of MSC into OA joints, the MSC group scored marginally lower than the HA group. This was however not statistically significant. In another study conducted in goats, intra-articular autologous mesenchymal stem cells (aMSC) injections resulted in only minor improvement to the disease progress in osteoarthritis, which was also not statistically significant[13]. In this direct comparison, it appears that the use of autologous or allogenic MSCs does not provide significant advantage. Nevertheless, to compare two studies which employ two different methodologies does not provide convincing evidence. To the best of our knowledge, there have not been any studies that have made side by side comparison of the efficacy of cartilage repair using allogenic and autologous MSC sources. There also appear to be limited number of literature reporting the use of allogenic MSC in treating OA. In a study by Toghraie *et al.*[28] using OA rabbit model, the injection of allogenic MSC produced superior healing than those which were left untreated in the present study. There were obvious immunologic response observed within repaired tissue, and that allograft cell transplants appeared to be a very closely matched to those of native tissues. One possible reason for this may be due to the genetic similarity of laboratory rats. The other possible reason is that MSCs are less immunogenic and can therefore evade the host immune response[29]. This would in turn regenerate new tissues.

The use of HA-MSC as a combined injection to treat OA has also been studied, although none have compared the efficacy of HA-MSC with HA or MSC in a side by side analysis. Choong *et al.*[30] have described that, during intra-articular injection of HA-MSCs, HA did not affect the characteristics of MSCs. Instead, the transformation of MSCs to functional lineage committed cells e.g. chondorcytes, were the result of the local tissue enviroment. Furthermore, the cell proliferation characteristics observed when MSCs are placed in HA suggests that HA regulates MSCs expansion and prevent MSC over growth. This may explain why the effects of MSCs in the HA-MSC suspension did not provide the superior regenerative ability as expected. Instead, the combination of both limits the potential of MSCs to repair damaged cartilage, hence in the present study the scores in the HA-MSC group were lower than those of HA and MSC alone. Regardless, this combination does exhibit positive effects as demonstrated in the present study, albeit lower than the HA or MSC groups. Other studies which used

HA-MSC also reported positive outcomes. Grogoli *et al*.[31] revealed that OA defects created using ACLT method in a rabbit model underwent hyaline-like cartilage regeneration after the implantation of MSC-HA. Similarly, Lee *et al*.[17] demonstrated that the use of HA-MSC provided significant healing to damaged cartilage than those which did not receive this treatment.

Considering the fact that there is limited repertoire of effective pharmacological interventions in the management of OA, the use of HA and MSC appears promising as it is less invasive and not cost prohibitive. Nonetheless, a significant body of clinical data supporting the beneficial effects of HA[32-34] and MSCs[31] seems not to be as convincing as most would wish for. Although some clinical studies have failed to show significant clinical benefit, they showed significant improvements from their baseline measures following the use of HA[35-37] and MSCs [12,17]. The results of the present study and of those previously reported appear to support the role for HA or MSCs as a treatment for OA rather than the combination of both. Although significant improvements were seen in HA treated group than that of the other groups, this comparison is rather limited as it was observed within a short period. Other limitations to the present study includes the few numbers of rats used in each group i.e. 7 rats per group and, the lack of observational time points. These would have provided more convincing results as increasing the number of samples and time points would verily narrow the confidence interval obtained. In the present study, the comparison in O'Driscoll score between day 18 and day 60 in the HA-MSC group was deemed significant as the mean comparative analysis demonstrated a *P* value of 0.03 (significant was set at $P<0.05$). It was therefore concluded that the drop in the values observed was significant and therefore HA-MSC did not stop the progression of OA. This would have been different if the significant *P* value was set at 0.01. However, in order to do this, the sample size has to be very much larger and therefore not applicable for this study. The use of more objective and sophisticated outcome measures, which may include functional analysis e.g. biomechanical assessments, extracellular protein analysis e.g. glycosaminoglycan content and, gene expression analysis e.g. COMP, may have provided a better measure of the efficacy of HA and MSC. However, these analyses require specialized laboratory skills, equipments and financial support; all of which were not available to the authors at the time the study was conducted. With all these issues, it is only appropriate to make it clear that the present study is preliminary and may serve as only an indicator of the outcome of a larger and more comprehensive study. Future studies to provide a stronger case for the use of HA and MSC independently to treat OA needs to be undertaken.

In conclusion, the findings of this study suggest that the use of either HA or MSCs effectively reduces OA progression better than their combined use. However, larger groups and longer periods of study are required to prove the robust findings of this study.

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